EXHIBIT A

PATENT
SCRIP1100

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	M. Von Herrath	Art Unit:	1636
Application No.:	09/336,672	Examiner	W. Sandals
Filed:	June 17, 1999		
Title:	COMPOSITIONS AND METHODS FOR THE TREATMENT OR PREVENTION OF AUTOIMMUNE DISORDERS		

#13

Commissioner of Patents
Washington, D.C. 20231

DECLARATION OF APPLICANT UNDER 37 C.F.R. §1.132

Sir:

I, Matthias G. von Herrath, M.D., inventor of the above-identified application, do hereby declare and state that:

1. I am familiar with the content of the above-identified application, including the methods for treating or preventing diabetes in a subject having or at risk of having the disorder contained in the Specification therein.

2. Experiments have been conducted under my supervision and control in the laboratories of The Scripps Research Institute, Department of Neuropharmacology, Division of Biology, in La Jolla, California, using the methods and procedures disclosed in the above-identified application to illustrate the efficacy of the invention methods for treating or preventing autoimmune diabetes in *nod* mice, an animal model for spontaneous autoimmune diabetes.

3. In the first experiment, tests were conducted to determine the effect of induced peripheral expression of self-antigen (porcine insulin B chain) (InsB) on spontaneous occurrence of IDDM in *nod* mice. Mice used in the experiments were female *nod/scid* mice obtained from Jackson Laboratories or *nod* mice bred from *Nod*/LtJ breeders (Taconic Farms) in which diabetes occurs spontaneously in 80% of the females and 30% of the males by 30 weeks of age.

The mice were vacciniated with a plasmid engineered to express porcine insulin B chain DNA under the control of the initial-early promoter of CMV. The plasmid is commercially available, widely used and described previously by Coon et al., *J. Clin. Invest.*, 1999, 104:189; Yokoyama et al., *J. Virol.*, 1995, 69:2684. The IE-CMV promoter has previously been shown to support transcription of various antigens in mammalian cells. The plasmid was expanded and purified from *E. coli*, using the Quiagen method. The mice were administered the plasmid expressing the self-antigen at the age of 7 days (gluteal muscle) with booster innoculations at 4 and 8 weeks (quadriceps muscle), bilaterally, at a total dose of 100μg in 100μl of sterile PBS. The blood glucose was monitored every two weeks beginning at 10 weeks of age.

Statistical analysis of the results of the blood glucose tests shows that priming at the age of 7 days and boosting at 4 and 8 weeks affected the kinetics of disease in female *nod* mice in an antigen-specific manner. Inoculation of InsB-expressing plasmid significantly slowed down the kinetics of disease (Figure. 1; Table 1 (attached), p log rank test < 0.001). By the age of 30 weeks, only around 35% of mice displayed full-blown disease in contrast with the expected rate of 80% in naïve female *nod* mice. The failure of pCMV control plasmid to aggravate the disease (Figure. 1) argues against, but does not exclude, conflicting effects of antigen expression and Th1-inducing unmethylated CpG bacterial motifs on disease kinetics. On the other hand, these tests show that immune stimulatory or inhibitory DNA motifs do not mediate by themselves the noted effect of pInsB on disease, since pCMV control plasmid failed to significantly modify the disease kinetics (Figure. 1).

To quantitate the effect of vaccination upon the *nod* mice, an additional study was designed to analyze the effect of vaccination upon the autoreactive T cell repertoire in the treated mice. To this end, an ELISA-based analysis was employed as follows.

4. *T cell bioassay*

In nitrocellulose wells precoated with anti-IFNγ, anti-IL-4 or anti-IL-10 rat anti-mouse cytokine antibodies (PharMingen), splenocytes or cells separated from local lymph nodes of vaccinated *nod* mice were stimulated *in vitro* with mitomycin-treated syngeneic antigen-presenting cells in the presence of InsB or GAD 65 peptides. Generally, the assay was carried out using serial dilutions of cell suspension, ranging from $4x10^5$ to $5x10^4$ responder cells/well. The number of stimulator cells was $2x10^5$ cells/well. After 72 hours, the assay was developed using sequential steps of washing, incubation with biotin-conjugated anti-cytokine antibodies (PharMingen), streptavidin-horse radish peroxidase and insoluble substrate (AEC). In certain cases, the responder splenocytes were subjected to a preliminary round of antigen expansion for 5 days, followed by a 3-day incubation with rIL-2 (20 U/ml) and concluded with 72 hour stimulation with peptides (20μg/ml) in the presence of feeder cells. Alternatively, the cells were stimulated with a mixture of 3μg/ml anti-CD3 and 1μg/ml anti-CD28 monoclonal antibodies (PharMingen). By this method and taking into account the possibility that a significant number of autoreactive T cells may differentiate *in vitro* toward cytokine-producing cells, the frequency of spot-forming cells in naïve and plasmid-immunized female *nod* mice was estimated.

The results of this study (Figure 2) show that early immunization with pInsB followed by two boosts at 4 and 8 weeks resulted in long-lasting expansion of GAD and InsB-specific T cell pool committed toward IL-4, but not IFN-γ production (Figure 2), compared with that in the spleens of diabetes-free (i.e. naïve) 30-weeks old mice. Thus, vaccination of female *nod* mice with pInsB triggered modifications in the T cell profile. The frequency of cytokine-producing T cells was on the order of tens/hundreds per million stimulator cells, approximately one/two orders of magnitude smaller than the frequencies of specific T cells usually triggered by viral infection.

Upon activation and differentiation, the T cells from pancreatic lymph nodes (PLN) and possibly other lymphoid organs migrate to the pancreas. Therefore, a further study was designed to assess the cytokine pattern of pancreas-infiltrating T cells in the vaccinated mice.

5. *Isolation of pancreas infiltrating T cells and ELISA*

T cells were released from pancreatic tissue by collagenase digestion and incubated for 24 hours with or without anti-CD3+anti-CD28 mAbs as follows. Pancreas tissue was digested for 45 minutes with 0.1mg collagenase / ml of 0.5% BSA-DMEM medium at 37°C. The resulting fragments were passed through 70-μm Falcon strainers and after washing, the interstitial cells including lymphocytes were isolated by Ficoll-Hypaque gradient centrifugation (15 minutes and 2,000 RPMI at RT). The cells were incubated over night (duplicate wells corresponding to individual pancreas organs) in HL-1 medium with or without stimulation using a mixture of anti-CD3+anti-CD28 mAbs (5+1μg/ml). The supernatants were harvested and the cytokine production determined by ELISA (IFNγ, IL-4, IL-1β, IL-10 US, TNF-α (BioSource Int.); TGF-β1 (R&D Systems).

The results of this experiment (Table 2) show that, in naïve mice that failed to progress to full blown disease with age, there was a statistically significant reduction in cytokine production by infiltrating T cells, affecting production of IL-1β, IFN-γ, and regulatory cytokines TGF-β and IL-10. By contrast, as shown in Table 2, in pInsB-vaccinated mice that did not develop diabetes by 30 weeks of age, infiltrating T cells displayed increased production of IL-4 and TGF-β1, compared to naïve or control plasmid-inoculated mice. Furthermore, the infiltrating T cells from pInsB-vaccinated mice displayed reduced production of IL-1β. The levels of TNFα were modest, probably due to the predominance of membrane-bound isoform.

Thus, these experiments show that administration to female *nod* mice of a plasmid containing DNA encoding bovine insulin B chain triggered a modified autoreactive T cell profile consisting in a shift from Th1 to Th2 immunity in spleen. This result was reflected in a decreased production of pro-inflammatory cytokines and increased regulatory cytokine secretion by pancreas infiltrating T cells. In addition, plasmid-mediated vaccination induced a process of intermolecular epitope spreading (pInsB:InsB=>GAD). this cytokine profile indicates that in protected mice that were treated according to invention methods a modified autoreactive T cell

profile was triggered consisting in a shift from Th1 to Th2 immunity, a positive regulatory immune response.

6. *Inoculation with InsB and IL-4*

In yet another experiment, using techniques substantially as described above, nod mice were immunized intramuscularly with a control plasmid (100μg/dose of pCMV control) or with a mixture of two plasmids (50μg pCMV-InsB and 50μg pHTLV-IL-4, expressing insulin B and IL-4 cytokine, respectively at the age of 7 days, with booster administrations at 4 weeks and 8 weeks. The blood glucose of the nod mice was monitored every two weeks beginning with 10 weeks of age and continuing through 18 weeks of age. The number of mice per group: n=19 (naïve); n=13 (pCMV) and n=10 (pCMV-InsB+pHTLV-IL-4).

The results of this study (Figure 3 attached) show that co-administration of polynucleotides encoding InsB and IL-4 substantially reduced the percentage of nod mice that developed the symptoms of diabetes as compared with naive non-obese diabetic mice.

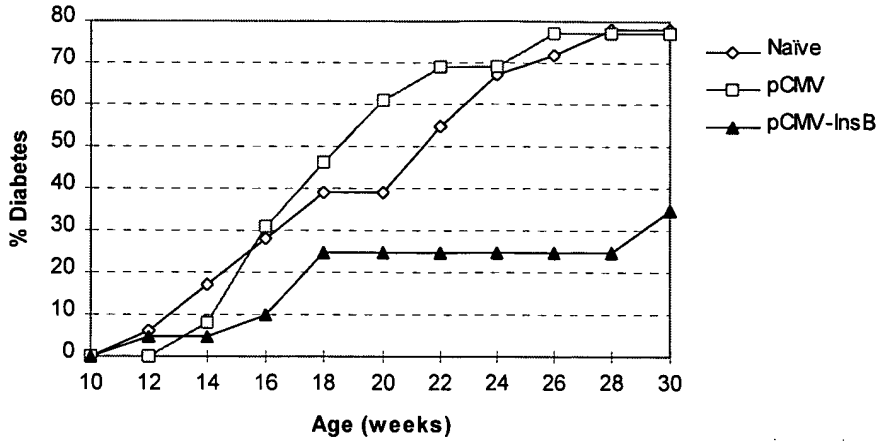
7. I further declare that all statements made herein of knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

12/21/2000
Date

Matthias G. von Herrath, M.D.

Attachments
Figures 1, 2 and 3 and Table 1.

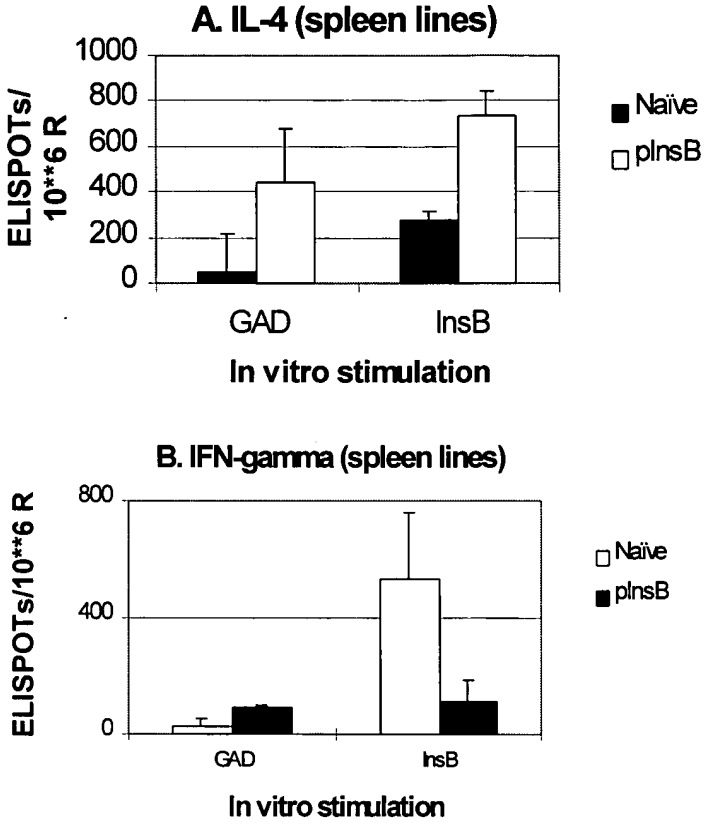
Fig. 1. Kinetics of IDDM in *nod* mice immunized with pInsB plasmid



Legend:

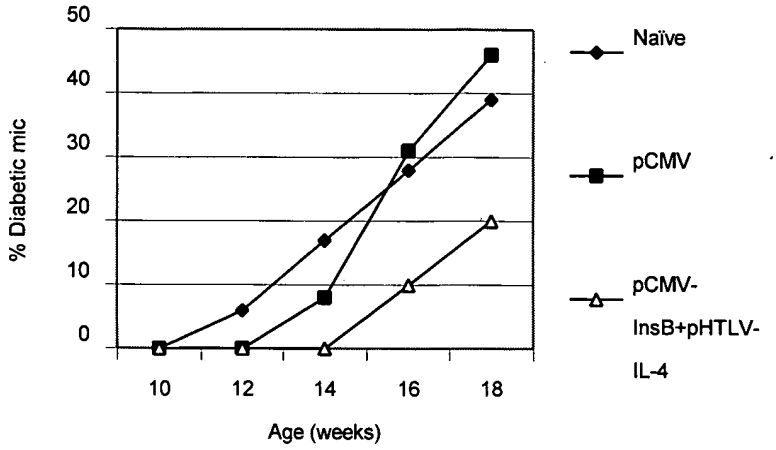
Mice were immunized with plasmids (100μg/dose) at the age of 7 days, 4 weeks and 8 weeks. The blood glucose was monitored evry other two weeks beginning with 10 weeks of age. Statistical analysis showed a statistically significant delay in the onset of disease in nod females immunized with pCMV-InsB (p of log-rank test < 0.005) and a near-significant decrease in the rate of disease in males immunized with pCMV-GAD65 (p of Fisher's test = 0.08).

Fig. 2. The cytokine profile of autoreactive T cells from spleens of DNA-vaccinated female *nod* mice



Legend:
Female nod mice were sacrificed at the age of 35 weeks; splenic cells were in vitro stimulated for 5 days with antigens, for 3 days with 10U/ml of rIL-2, followed by 72 hour-stimulation with peptides in the presence of feeder cells. The ELISPOT analysis was carried out as previously described; background was subtracted.

Figure 3: Co-administration of antigen and cytokine-expressing plasmid delays the onset of spontaneous autoimmune diabetes in *non-obese diabetic* mice



Legend:

Mice were immunized intramuscularly with plasmids (100μg/dose of pCMV control or a mixture of 50μg pCMV-InsB and 50μg pHTLV-IL-4 expressing insulin B and IL-4 cytokine, respectively) at the age of 7 days, 4 weeks and 8 weeks. The blood glucose was monitored evry other two weeks beginning with 10 weeks of age. Number of mice / group: n=19 (naïve); n=13 (pCMV) and n=10 (pCMV-InsB+pHTLV-IL-4).

Table 1. Effect of DNA-mediated delivery of InsB self-antigen on IDDM (statistical analysis)

Group	Overall rate of IDDM		Kinetics of disease	
	Diabetes rate	Significance (p chi-square)	Time to 50% diabetes rate	Significance (p log-rank test)
Naïve	12/18 (67%)	-	<22w	-
pCMV	10/13 (77%)	>0.05	<20w	>0.05
pCMV-InsB	5/20 (25%)	0.0057	>28w	<0.001

Table 2. Cytokine production (pg/ml) by infiltrating T cells from 30weeks old non-diabetic *nod* female mice

Group	IL-4	IL-10	TGF-β1	IFN-γ	IL-1β
Naïve	5±3	1±1	17±6	30±22	20±5
Naïve (11-12w)	0	**7±2**	**64±21**	**74±16**	**135±61**
pCMV	3±2	0	17±11	50±45	59±46
pCMV-InsB	**19±4***	1±1	**32±10**	25±12	**12±3**

*Significantly different from naïve.